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# CELLULAR AND NETWORK MECHANISMS IN NEURODEGENERATIVE DISORDERS – NEUROTOXICITY AND RESCUE STRATEGIES

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# Cellular and Network Mechanisms in Neurodegenerative Disorders – Neurotoxicity and Rescue Strategies

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

# ABSTRACT

The brain can be described as a complex network and its functioning depends on an efficient communication between all its components. Large-scale communication is made possible by neuronal-network oscillations. Oscillations in the gamma-frequency range (30-80 Hz) are associated with cognitive functions such as attention, working memory, sensory perception and long-term memory encoding and recall. These oscillations occur in the neocortex and hippocampus, and are known to be impaired in many diseases displaying cognitive-deficit symptoms, including neurodegenerative diseases. In the studies contained in this thesis we investigate basic neuronal mechanisms involved in the generation of gamma oscillations and their disruption in models of Alzheimer's disease and Parkinson's disease. Moreover, acting pharmacologically on different pathways, we try to prevent and/or rescue the impairment of the cognition-relevant rhythm and its behavioral consequences.

In paper I we investigate whether the deleterious effects of amyloid-β peptide on cellular, network and behavioral level can be either prevented or rescued by targeted activation of the proteasome through the modulation of calcium dynamics. The use of mouse hippocampal slices, *Drosophila* fly models and induced pluripotent stem cells from AD patients showed that the inhibition of T-type calcium channels could be an effective therapeutic approach for AD and, potentially, other amyloidogenic brain diseases.

In paper II we study brain hypometabolism and insulin resistance, both known to be risk factors for and common outcomes of amyloid-β peptide accumulation. The synergistic use of pyruvate, as an alternative source of energy, and insulin, to counteract insulin resistance, is efficient in rescuing and preventing synaptic and network dysfunction induced by acute application of amyloid-β peptide on mice hippocampal slices.

In paper III we examine the role of histamine as a potential rhythmogenic neurotransmitter. In rat hippocampal slices the perfusion of histamine generates transient dose-dependent gamma oscillations, and this action seems to be dependent on the H1 receptor. Our data suggest that the generation of gamma oscillations may depend on H1 receptor-mediated inhibition of KCNQ channels.

Lastly, in paper IV we study network activity and behavior of a Parkinson's disease mouse model. The striatal injection of 6-hydroxidopamine disrupts the endogenous circadian rhythm of mice, reduces their motor activity and degrades gamma oscillations. Systemic administration of a histamine H3 receptor antagonist rescues normal rest/activity cycle and memory impairment underlain by gamma oscillations disruption.

# LIST OF SCIENTIFIC PAPERS

I. **Papadia D**, Marks C, Romero N, Balleza-Tapia H, Shahsavani M, Johansson J, Falk A, Acebes A, Altun M, Fisahn A. T-type calcium channel inhibition as a novel therapeutic target for amyloid diseases in the brain - Aβ aggregates clearance and rescue of neuronal function in animal and human models of Alzheimer's disease. (Manuscript)

II. **Papadia D**, Zilberter M, Domènech-Estevez E, Chen G, Chrast R, Johansson J, Fisahn A. Combination treatment for insulin resistance and hypometabolism rescues hippocampal neuron function and network gamma oscillations from Aβ-induced impairment. (Manuscript)

III. Andersson R, Galter D, **Papadia D**, Fisahn A (2017) Histamine induces KCNQ channel-dependent gamma oscillations in rat hippocampus via activation of the H1 receptor. *Neuropharmacology* 118, 13-25

IV. Masini D, Lopes-Aguiar C, Bonito-Oliva A, **Papadia D**, Andersson R, Fisahn A, Fisone G (2017) The histamine H3 receptor antagonist thioperamide rescues circadian rhythm and memory function in experimental parkinsonism. *Translational Psychiatry* 7, e1088

# PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Samokhina E, Popova I, Malkov A, Ivanov AI, **Papadia D**, Osypov A, Molchanov M, Paskevich S, Fisahn A, Zilberter M, Zilberter Y (2017) Chronic inhibition of brain glycolysis initiates epileptogenesis. *Journal of Neuroscience Research* 95, 2195-2206

II. Balleza-Tapia H, Crux S, Andrade-Talavera Y, **Papadia D**, Chen G, Johansson J, Fisahn A. Trpv1 receptor activation rescues hippocampal neuron function and network gamma oscillations from Aβ-induced impairment. (Manuscript)

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 6-OHDA | 6-hydroxidopamine |
| Aβ | Amyloid-β |
| ACSF | Artificial cerebrospinal fluid |
| AD | Alzheimer's disease |
| AP | Action potential |
| APP | Amyloid precursor protein |
| CA | *Cornu Ammonis* |
| CNS | Central nervous system |
| DA | Dopamine |
| DG | Dentate Gyrus |
| EC | Entorhinal cortex |
| EEG | Electroencephalogram |
| EPSCs | Excitatory post-synaptic currents |
| FS-IN | Fast-spiking interneurons |
| GluT | Glucose transporters |
| HD | Huntington disease |
| IEI | Inter-event-interval |
| IN | Interneurons |
| ING | Interneuron gamma |
| iPSCs | Induced pluripotent stem cells |
| IPSCs | Inhibitory post-synaptic currents |
| KA | Kainate |
| LFP | Local field potential |
| MCI | Mild cognitive impairment |
| PC | Pyramidal cell |
| PD | Parkinson's disease |
| PING | Pyramidal interneuron gamma |
| PSEN1 | Presenilin 1 |
| PSEN2 | Presenilin 2 |

| | |
|---|---|
| $R_{in}$ | Input resistance |
| RMP | Resting membrane potential |
| SNpc | *Substantia nigra pars compacta* |
| UPS | Ubiquitin-proteasome system |
| VTA | Ventral tegmental area |

# 1 INTRODUCTION

## 1.1 Overview of the hippocampal formation

The hippocampus is a complex and fascinating brain region with enormous clinical significance. It is a small structure, part of the limbic system, located in the medial temporal lobe of the human brain and is of great importance for memory formation, learning, spatial navigation and cognition.

The hippocampus has a distinctive curved shape that has been likened to the sea-horse monster of the Greek mythology, hence the name. The hippocampal cortex is composed of Dentate Gyrus (DG) and *Cornu Ammonis* (CA, also known as *hippocampus proper*), which is divided into 3 main regions: CA1, CA2, CA3 (**Figure 1**). The DG is a structure important for episodic memory formation (Nakashiba et al., 2008) and consists mostly of glutamatergic granule cells tightly packed to form a granular layer capping the *hippocampus proper*. It receives glutamatergic input from the Entorhinal cortex (EC) through the Perforant Path (**Figure 1**). In turn, cells in the DG give rise to axons (mossy fibers) forming synapses with pyramidal cells (PC) in the CA3 region: this region is of particular interest for its role in the consolidation of episodic and contextual memories (Nakashiba et al., 2008; Jadhav and Frank, 2009). CA3 pyramidal cells send excitatory inputs to the pyramidal neurons in CA1 through a branch of axons called Schaffer collaterals. Together, these connections form the famous trisynaptic excitatory pathway within the hippocampus, described originally by Per Andersen (Andersen, 1975). In addition, many CA3 pyramidal cells also have recurrent connections. CA1, finally, is the main output of the hippocampus, sending projections back to the EC and the *subiculum* (Amaral et al., 1989), and has high importance in the formation of spatial memories (Tsien et al., 1996). CA2 is the least studied among the hippocampal regions and it seems to be involved in the formation of social memories (Hitti & Siegelbaum, 2014).

The hippocampus, in addition, receives several subcortical modulatory inputs: from the tuberomammillary nucleus (histamine), raphe nuclei (serotonin), locus coeruleus (norepinephrine), ventral tegmental area (dopamine) and medial septum (acetylcholine).

The stratification of the CA area (**Figure 1**) is largely the same in the different subfields (CA1-CA3), with the outermost layer being the *alveus*, containing solely fibers. The next layer is the *stratum oriens*, containing inhibitory interneurons (IN) and the basal dendrites of pyramidal cells. Further inwards we find the *stratum pyramidale*, containing glutamatergic pyramidal cells. The following layer, *stratum radiatum*, contains inhibitory interneurons and associational connections within CA3 and between CA1 and CA3. The innermost layer is the *stratum lacunosum-moleculare*, containing the termination of pyramidal cells apical dendrites and axons from the EC. The main difference in the stratification between the different

subfields is that CA3 has an additional layer, *stratum lucidum*, containing the mossy fiber axons from the DG, and placed in between *stratum pyramidale* and *stratum radiatum*.

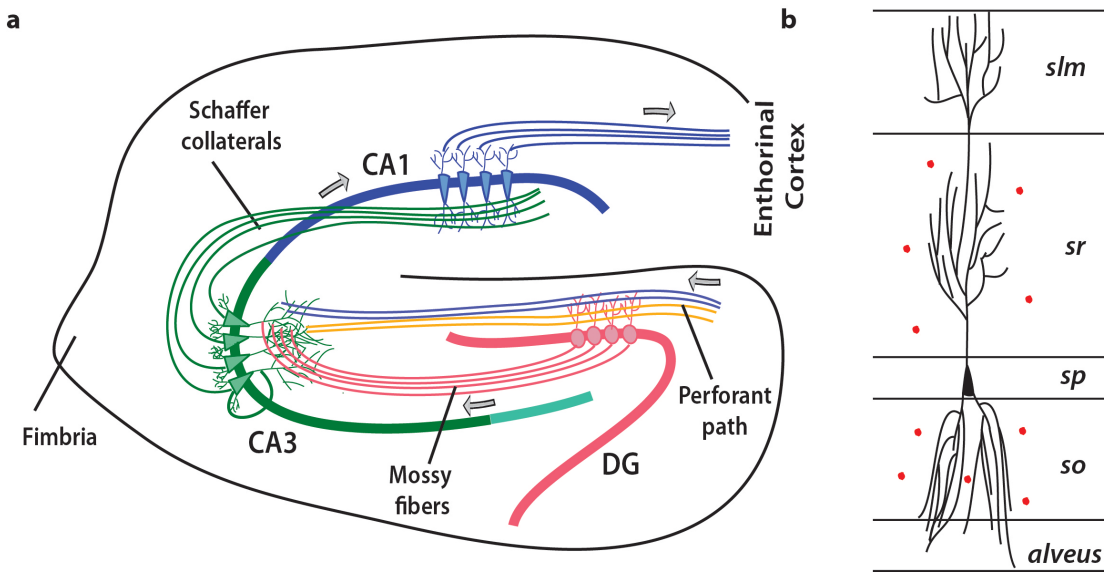


**Figure 1**. Schematic of the trisynaptic excitatory pathway (**a**). Axons from layers II (yellow) and III (purple) in the Entorhinal Cortex (EC) project to the Dentate Gyrus (DG) through the Perforant Path. DG sends projections to CA3 pyramidal cells through mossy fibers. CA3 in turn project to CA1 pyramidal cells through Schaffer collaterals. CA1 send projections back to the EC. In **b** schematic representation of the stratification of the CA area: *stratum oriens* (*so*) receives afferents from CA3 and contains interneurons (red dots); *stratum pyramidale* (*sp*) contains the cell bodies of pyramidal cells; *stratum radiatum* (*sr*) receives afferents from CA3 and contains interneurons (red dots); *stratum lacunosum-moleculare* (*slm*) receives afferents from Entorhinal Cortex.

The hippocampus is thought to be the place where memories are laid down. The relation between hippocampus and memory derived from a famous report by Scoville and Milner (Scoville et al., 1957): they described the results of surgical removal of the hippocampus in a patient named Henry Molaison, in an attempt to relieve him from severe epileptic seizures. The outcome of the surgery was an unexpected and severe amnesia. From then on the hippocampal formation has been widely studied in its connection to long-term memory. The hippocampus, moreover, is the structure where synaptic plasticity was first discovered (Bliss & Lømo T, 1973). It has also been extensively studied for its role in spatial memory and navigation. O'Keefe and Dostrovsky originally promoted the spatial theory after their observations in 1971 (O'Keefe J & Dostrovsky J, 1971), stating that certain cells in the hippocampus ("place cells") are activated when a rat occupies a certain place in the environment.

## 1.2 Brain oscillations

The term "brain oscillations" refers to rhythmic electrical activity generated spontaneously and in response to stimuli in the central nervous system (CNS; Basar, 2013). The discovery of brain oscillations is credited to Hans Berger, who recorded the first electroencephalogram (EEG; Berger, 1929). Many different structures in the CNS are known to be able to generate such rhythms. Among these we can find: all cortical areas, cerebellum, olfactory bulb, and hippocampus (Gray and Singer, 1989; Hartmann et al., 1998; Beshel et al., 2007; Gray, 1994). Brain oscillations exhibit a wide range of frequencies (from 0.05 to 500 Hz), and they can be grouped in bands within the same neuronal network associated with different brain states (Buzsáki and Draguhn, 2004).

The hippocampus is one of the structures exhibiting some of the most prominent forms of synchronous rhythmic activity in the CNS. Hippocampal oscillations have been widely studied for their implications in important higher processes in the brain, such as cognition, learning, memory and navigation (Winson, 1978; Seager et al., 2002). Oscillations derive from synchronous action potential (AP) firing of neurons that give rise to local field potentials (LFP) (Börgers and Kopell, 2003; Bartos et al., 2007; Klausberger and Somogyi, 2008; Atallah and Scanziani, 2009) and they can be divided according to their frequency band into delta, theta, beta and gamma. Oscillations in different frequency bands can occur simultaneously and, according to their relation, they can represent different behavioral states (Gloveli et al., 2010); this coordinated activity seems to play a critical role in memory encoding (Lisman, 2005).

Delta oscillations (0-4 Hz) are associated with slow-wave sleep (Happe et al., 2002; Headley and Paré, 2016); theta oscillations (6-10 Hz) are related to locomotor activities defined as voluntary, preparatory or exploratory (Vanderwolf, 1969; Buzsáki, 2002); beta oscillations (12-30 Hz) are involved in exploration of new environments and movement planning (Murthy and Fetz, 1996; Grossberg, 2009). Finally, and most importantly for the scope of this thesis, gamma oscillations (30-80 Hz) are fast oscillations associated with cognition, learning, memory and attention (Singer, 1993; Buzsáki et al., 2003). Oscillations in the gamma frequency band are dependent on precise timing of APs generation and the finely balanced interplay between excitatory and inhibitory neurotransmission in the neuronal network (Csicsvari et al., 2003; Atallah and Scanziani, 2009; Kurudenkandy et al., 2014).

Gamma oscillations are disrupted in several brain disorders associated with cognitive decline, like epilepsy, depression and schizophrenia (Benedek et al., 2016; Liu et al., 2014; Spencer et al., 2008; McNally et al., 2016) and in many neurodegenerative diseases, such as Alzheimer's disease (Goutagny and Krantic, 2013; Kurudenkandy et al., 2014; Nakazono et al., 2018) and Parkinson's disease (Weinberger et al., 2009).

### 1.2.1 Generation of hippocampal gamma oscillations

Gamma oscillations in the hippocampal formation can be generated either in area CA3 or in the DG (Csicsvari et al., 2003). The recurrent excitatory connections in CA3 mentioned previously and the perisomatic inhibitory connections (Fisahn et al., 1998) are the perfect features allowing this area to sustain its own rhythmic activity without external input.

Two mechanistic explanations have been proposed for the generation of gamma oscillations: Interneuron Network Gamma (ING) and Pyramidal Interneuron Network Gamma (PING). The ING model suggests that GABAergic neurons (interneurons) are reciprocally connected and that they are sufficient to generate and maintain the rhythmic activity thanks to their mutual synaptic inhibition and excitation through gap junctions connections (Hormuzdi et al., 2001). This is therefore the minimal circuit needed to generate gamma oscillations if interneurons are provided with an initial strong and phasic excitatory drive. It is likely, in this model, that different classes of interneurons have different involvement in the generation of gamma oscillations (Whittington et al., 2000; **Figure 2**). Pharmacological manipulation of metabotropic glutamate receptors in hippocampal slices induced oscillations that persisted in absence of excitatory stimuli (Whittington et al., 1995), providing support for the ING model. However, ING alone is unlikely to represent a specific model of information processing in the CNS, as excitatory cells (i.e. pyramidal cells) are integral part of the cortical network. The PING model, on the other hand, proposes that gamma oscillations can only arise from the interplay between interneurons and pyramidal cells. In this model pyramidal cells send excitatory inputs to interneurons, which in turn inhibit pyramidal cells setting specific time windows in which they can fire. According to this model, therefore, the interplay between PC and IN is essential to generate and maintain the oscillations (Fisahn et al., 1998; Whittington et al., 1997; Atallah and Scanziani, 2009; Buzsáki and Wang, 2012).

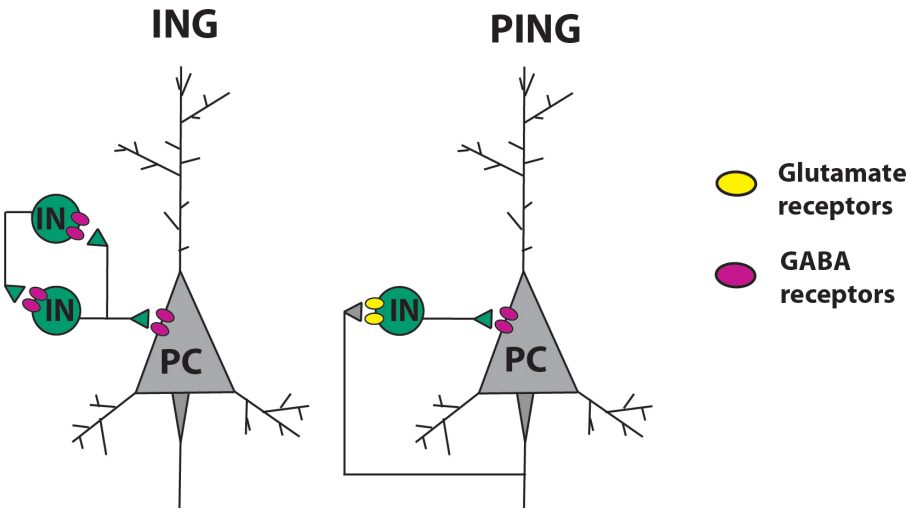


**Figure 2**. Schematic of the interneuron network gamma (ING) and pyramidal interneuron network gamma (PING) models.

### 1.2.2 *In vitro* models of hippocampal gamma oscillations

Several *in vitro* models of hippocampal gamma oscillations have been developed to permit the study of cellular and synaptic mechanisms in network activity. Alive hippocampal slices maintain many aspects of *in vivo* biology, with preserved architecture and local synaptic circuitry, but at the same time they also allow for precise control of the extracellular environment (Cho et al., 2007). Hippocampal slices can be used to reproduce gamma oscillations using pharmacological or electrical stimulation (Traub et al., 1996) to provide excitation to the network. Pharmacologically, they can be induced by activation of metabotropic glutamate receptors (Whittington et al., 1995; Gillies et al., 2002), muscarinic acetylcholine receptors (Fisahn et al., 1998; Buhl et al., 1998; Hajos et al., 2004) or kainate receptor (Buhl et al., 1998; Fisahn et al., 2004). Local gamma oscillations can also be classified as transient or persistent. Kainate (KA), by triggering a generalized depolarization, and carbachol, by activating the cholinergic system, provide stable and reliable oscillations for hours and, therefore, they are useful to explore the mechanisms underlying the physiology of oscillations or to modulate them pharmacologically and study the contribution of individual neurons. Transient forms of gamma oscillations (lasting for a few seconds or minutes at most) can be evoked *in vitro* by tetanic stimulation (Whittington et al., 1997), through pressure ejection of glutamate (Pöschel et al., 2002) or high molarity of potassium (LeBeu et al., 2002). Recently, we found a novel and physiological mechanism to induce transient gamma oscillations that is described in **Paper III** in this thesis: the activation of histamine receptor H1 elicits transient form of gamma oscillations in area CA3 of rats hippocampus, by rendering both PC and fast-spiking interneurons (FS-IN) more excitable.

The possibility to reproduce gamma oscillations *in vitro* provides a unique tool that can be used to better understand the physiology of the brain and the mechanisms responsible for the cognitive decline caused by several brain disorders.

## 1.3 Neurodegenerative diseases

Disruption of gamma oscillations has been observed in several neurological conditions and neurodegenerative disorders. Such disruption, as said before, is strictly related to the cognitive decline associated with neurodegenerative disorders. In addition to neuronal, network and cognitive functions decline, many neurodegenerative disorders also manifest the hallmarks of protein misfolding and aggregation as well as formation of inclusion bodies. In this thesis, the focus lies on Alzheimer's disease (AD) and Parkinson's disease (PD).

### 1.3.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia in the elderly and affects more than 40 million people worldwide. AD is a progressive neurodegenerative disorder, which results in the loss of neurons, mainly in the cortex and hippocampus (Nussbaum and

Ellis, 2003). It is also a socially disruptive disease, affecting individuals who may live for a long time with clinical symptoms and, therefore, represents a socio- and economic- burden. This has led to increasing efforts from the scientific community to find therapeutic agents able to prevent its progression. Despite this, AD research is still extremely fragmented, with no treatment, method of early diagnosis or unifying theory of pathogenesis currently available.

AD is a heterogeneous disorder with both familial and sporadic forms. Sporadic forms are the most common, accounting for up to 99% of the cases (Goedert and Spillantini, 2006). Familial AD has a very early onset and involves inheritance of a mutated form of Aβ cleaving or regulating genes: APP (amyloid precursor protein), PSEN1 (presenilin 1) or PSEN2 (presenilin 2). AD typically progresses in three general stages: early-stage (mild), middle-stage (moderate) and late-stage (severe). However, neuronal dysfunction seems to occur a decade or more before any clinical sign or symptom of the disease (preclinical stage) and a definitive diagnosis can be made only with post-mortem studies.

Since the first description of the disease by Alois Alzheimer is 1907, it has been clear that the neuropathological picture includes aggregates of amyloid-β peptide (now known as amyloid plaques) and tangled bundles of fibers of tau peptide (now known as neurofibrillary tangles or tau tangles): both amyloid plaques and neurofibrillary tangles are the hallmarks of the disease. For the purpose of this thesis, the focus will be on amyloid-β peptide (Aβ).

The amyloid cascade-hypothesis was originally proposed by Selkoe in 1991 (Selkoe, 1991; Hardy and Allsop, 1991) and now dominates AD research. It stipulates that toxic Aβ is one of the main culprits for the pathologic neuronal changes in the brain. According to this hypothesis the initiating event is the imbalance between the production and the clearance of Aβ, resulting in the accumulation of the peptide and leading to neuronal dysfunction, synaptic loss and cognitive decline in patients (Hardy and Selkoe, 2002; Walsh and Selkoe, 2007).

Aβ is produced from its transmembrane precursor, APP, through the sequential cleavage by β- and γ-secretase (Haass et al., 1992; Carter and Lippa, 2001; Haass et al., 2012). The γ-secretase cleavage can bring to two outcomes: most of the Aβ peptides produced are 40 amino acids long ($A\beta_{40}$); the less frequent and longer form is $A\beta_{42}$. This second form is more hydrophobic and neurotoxic than $A\beta_{40}$ and it is the main component of Aβ plaques (Carter and Lippa, 2001; LeVine, 2004). The Aβ monomers are soluble and known to aggregate extracellularly into oligomers and fibrils (Bucciantini et al., 2002; Crews and Masliah 2010; **Figure 3**).

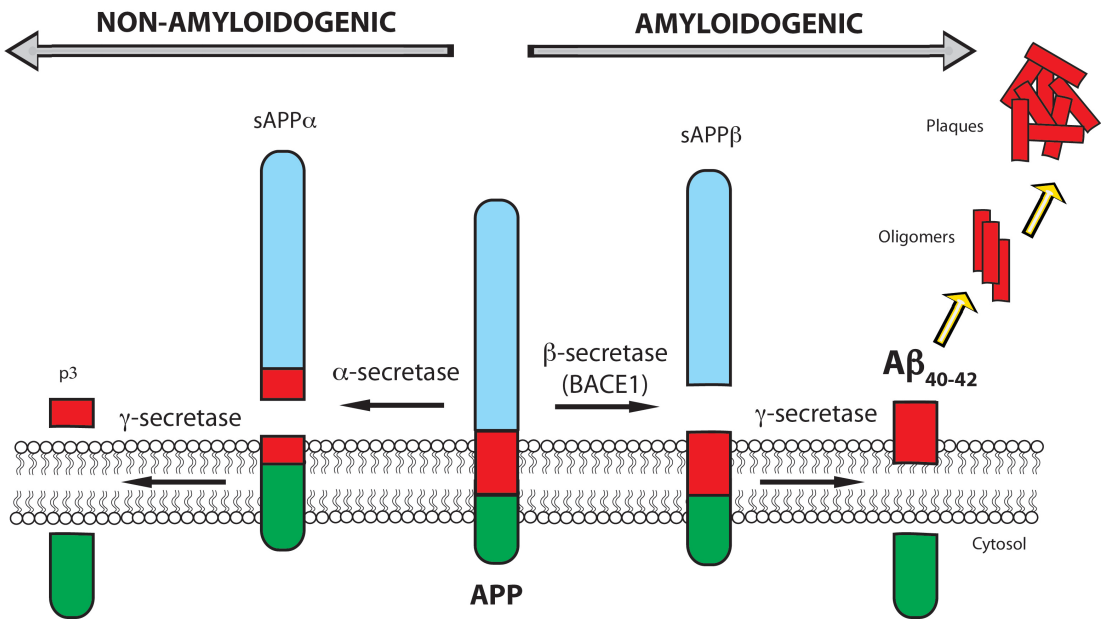


**Figure 3**. Schematic of the two pathways of APP processing. In the amyloidogenic pathway the β-secretase cleaves APP releasing sAPPβ and a fragment that is subsequently cleaved by γ-secretase to release $A\beta_{40\text{-}42}$. This accumulates, forming oligomers and plaques.

The progressive accumulation of Aβ in the brain (due to excessive production or failure in its clearance) initiates many secondary effects, such as insulin resistance (Xie et al., 2002; Biessels and Regan, 2015), decreased glucose consumption (Jin et al., 2013), activation of microglia and astrocytes (Barger and Harmon 1997; Streit, 2010), neuroinflammation (Salminen et al., 2009), dysregulation of calcium homeostasis (Small, 2009; Brawek and Garaschuk, 2014), neuron hyperactivity (Busche et al., 2008; Minkeviciene et al., 2009), oxidative stress (Cheignon et al., 2018) and synaptic failure (Bayer and Wirths, 2010; Reiners et al., 2016; **Figure 4**).

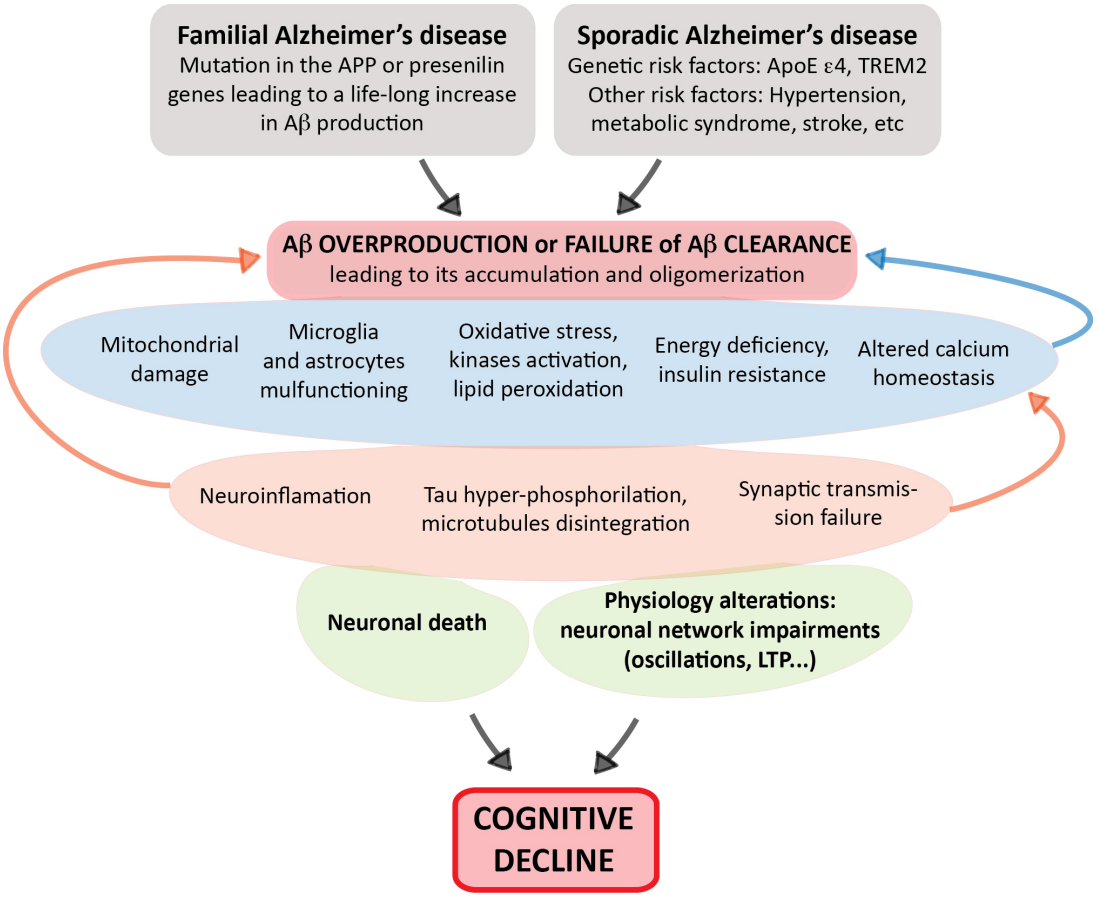


**Figure 4**. Schematic of amyloid-β cascade hypothesis. According to this hypothesis, the central event of the disease pathogenesis is the imbalance between Aβ production and clearance and many entangled downstream effects leading to cognitive decline. **ApoE ε4**: apolipoprotein E ε4; **TREM2**: triggering receptor expressed on myeloid cells 2; **LTP**: long-term potentiation.

Among the many Aβ effects, of importance for the scope of this thesis are the dysregulation of neuronal calcium homeostasis and hypometabolism. A hypothesis formulated by Chen and Nguyen suggests that the combination between energy and calcium signaling deficits is the explanation for all the hallmarks of AD (Chen and Nguyen, 2014).

It is well known that calcium signaling is one of the most important regulatory factors for neurons, being involved in many indispensable processes such as synaptic transmission, synaptic plasticity and genes transcription. Physiological calcium levels have also been proven to promote the non-amyloidogenic pathway in the APP processing (Bezprozvanny and Mattson, 2008). However, during normal aging, calcium levels increase in neurons (LaFerla, 2002; McInnes, 2013), and it has been long known that the accumulation of Aβ results in an abnormal raise of cytosolic calcium in the AD pathology (Mattson et al., 1992; Butterfield et al., 1994; Mark et al., 1995). The mechanism through which Aβ initiates the calcium influx in neurons has been widely studied (Arispe et al., 1993; Demuro et al., 2005; De Felice et al., 2007), but there is still no unifying theory. Actually, it is still debated whether the disruption of calcium homeostasis is a cause or a consequence of the disease. In

fact, it has also been described that upregulation of calcium levels, in turn, is stimulating the production of Aβ (Querfurth and Selkoe, 1994; Pierrot et al., 2006; Ahmad et al., 2009; Itkin et al., 2011). Independently of which comes first - Aβ accumulation or calcium dysregulation - they create a self-feeding cycle that leads to mitochondria impairment, synaptic signaling damage, neuronal apoptosis and, therefore, cognitive decline.

Another major effect of Aβ accumulation relevant for this thesis is disturbed cerebral glucose metabolism accompanied by insulin resistance. The human brain requires great amounts of energy, being one of the most metabolically active organs in the body, and its major source of energy is ATP derived from glucose. Disturbances in cerebral metabolic rate of glucose in AD patients have been described for the first time in 1983 by de Leon (de Leon et al., 1983). Since then, research on hypometabolism in AD has bloomed and, similarly to what has been said before for calcium, it seems to be both a cause and a consequence of Aβ accumulation (Gibson et al., 2010; Simoncini et al., 2014). Hypometabolism seems to start in memory-related brain regions (Mosconi, 2005) and correlates with cognitive impairment in AD patients (Pappatà et al., 2008).

It is also well known that insulin dynamics play a role in neurotransmission and cognition (Rhoads et al., 1984; Kern et al., 2001; Ahmadian et al., 2004; Ghasemi et al., 2013). Insulin receptors and insulin-sensitive glucose transporters are present in the brain and they are particularly abundant in brain regions involved in learning and memory, such as the hippocampus. Insulin resistance seems to promote the expression of APP and the deposition of Aβ (de la Monte, 2012) and can exacerbate an already existing AD pathology. Moreover, increased Aβ levels give rise to stronger insulin resistance (Xie et al., 2002; De Felice et al., 2014) creating yet another vicious cycle. However, it seems that insulin does now have a significant role in glucose uptake in the brain, and it might play other roles in glucose homeostasis (Blázquez et al., 2014).

The original amyloid cascade hypothesis indicated that the main event responsible for all the changes in AD brains was the accumulation of insoluble fibrillar Aβ in the extracellular space (Selkoe, 1991; Hardy and Allsop, 1991). This hypothesis was later slightly modified and the importance of soluble Aβ oligomers was also recognized. Moreover, newer theories suggest that intraneuronal Aβ plays an important role in neurotoxicity and in the synaptic pathology (Takahashi et al., 2002; Gouras et al., 2010; Mohamed and Posse de Chaves, 2011). One of the hypotheses is that Aβ accumulation starts intracellularly, leading to neuronal dysfunction (cytosolic disruption and mitochondria failure; Chen and Yan, 2007). This would lead to neuronal cell death and the consequent release of Aβ in the extracellular environment (Gouras et al., 2000; D'Andrea et al., 2001; Cuello et al., 2005). Another hypothesis is that Aβ is taken-up by neurons after its secretion to the extracellular space (Bayer and Wirths, 2010). There are several theories regarding the mechanism(s) through which the uptake occurs: it has been shown that the use of integrin antagonists enhances the Aβ internalization and the use of NMDA receptor antagonists blocks it (Bi et al., 2002). It has also been proven

that the α7 nicotinic acetylcholine receptor facilitates Aβ uptake (Nagele et al., 2002). Moreover, Li and colleagues (2007) suggested that there could be a passive diffusion through the membrane. It is clear, however, that both intracellular and extracellular Aβ are important in the progression of the disease and they seem to be connected by a dynamic relationship (Vetrivel et al., 2005; Crews and Masliah, 2010).

As mentioned previously, AD is associated with a dramatic decline in cognitive performance, which includes also the hippocampus-dependent memory. The main manifestation of the alterations of neuronal network patterns in AD patients is the so called "EEG slowing" and underlies their cognitive dysfunction (Kowalski et al., 2001). Clinical data show that this cognitive decline goes hand-in-hand with a decrease of neuronal network oscillations in the gamma frequency band (30-80 Hz; Singer, 1993). It is still debated whether the changes in these neuronal rhythms are secondary phenomena in the progression of the disease or a cause of the biological and functional changes happening during the progression of the disease. Iaccarino and colleagues (2016) have recently proven that optogenetical stimulation of the hippocampal region of 5XFAD and APP/PS1 mice (AD models) at gamma frequencies reduces Aβ levels. The restoration of gamma oscillations was shown to induce microglial morphological changes that promote microglial Aβ uptake. Moreover, through gamma stimulation, the processing of APP was altered, inducing a drastic decrease of Aβ generation (Iaccarino et al., 2016). Restoration of gamma oscillations is suggested to be a therapeutic strategy against Aβ load, even though the underlying mechanisms are yet to be described and the translation to human pathologies would still be a challenge.

Our lab has previously described the mechanisms underlying the degradation of gamma oscillations induced by Aβ, finding a desynchronization of AP firing of pyramidal cells and a shift in the excitatory/inhibitory currents balance (Kurudenkandy et al., 2014). In **paper I** and **II** different strategies turned out to be effective in preventing and rescuing those Aβ-induced cellular and network changes and, therefore, in restoring the power of gamma oscillations.

### 1.3.2 Parkinson's disease

Parkinson's disease (PD) is one of the most common age-related neurodegenerative disorders affecting 2-3% of the population older than 65 years of age. The first description of the disease as a neurological syndrome dates to 1817, when James Parkinson described its clinical features in "An Essay on the Shaking Palsy" (Parkinson J, 2002):

> *"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured".*

However, it was Jean-Martin Charcot, 50 years later, who described more in detail the disease and named it after Parkinson. In his description in 1872, he pointed out all the major

symptoms: resting tremor, bradykinesia, rigidity and the impairment in the ability to initiate movements (for review see Goetz, 2011).

From the first reports of the disease, a lot has been discovered on its pathophysiology, but the exact cause of PD is still unknown. For this reason, still only symptomatic treatments exist and nothing can be done to halt the neurodegeneration. Although most of the parkinsonian cases are sporadic (85-90%) studies on the rare familial cases have given great insight into the genetic factors involved in this disease. Many genes have been found to be implicated in the familial form of PD (Mhyre et al., 2012). Among these, PARK1/4, SNCA and MAPT (Mata et al., 2011) and LRRK2 (Simon-Sanchez et al., 2009) seem to be also involved in the sporadic form.

Even though PD is primarily diagnosed by its motor symptoms, these are preceded by non-motor symptoms, equally severe and disabling, including olfactory dysfunction, cognitive and affective deficits and sleep disorders (Langston, 2006). The disease can impact on different cognitive domains, affecting memory, naming, visuomotor and complex attention (Hanna-Pladdy et al., 2013). The memory deficits associated with the disease occur more frequently in the domains of verbal learning and delayed recall.

The principal pathological hallmarks of the disease are the progressive loss of dopaminergic neurons, starting from the *substantia nigra pars compacta* (SNpc), and the intracellular α-synuclein aggregates (called Lewy Bodies; first described by Lewy in 1912). The fact that PD patients also experience non-motor symptoms indicates that non-dopaminergic neurons are also affected by the disease (Chaudhuri et al., 2006). It seems that pathologic changes precede clear outward symptoms by two decades or more (Gazewood et al., 2013): by the time symptoms occur, already 60-70% of the neurons in the SNpc have died. The loss of such a great amount of these pigmented neurons is the reason why it can be appreciated visually. A question that remains open is why the disease affects primarily dopaminergic neurons in the SNpc. Dopamine (DA) is involved in complex brain functions including voluntary movement and goal-directed behavior, cognition, working memory and wakefulness (Grace et al., 2007; Redgrave et al., 2010; D'Ardenne et al., 2012; Arnulf and Leu-Semenescu, 2009). The majority of the DA neurons are located in the SNpc as mentioned before, and in the Ventral Tegmental Area (VTA). According to their location, DA neurons have different electrophysiological properties and a specific gene expression pattern (Roeper, 2013): neurons in the SNpc are involved in motor functions while the ones in the VTA support cognitive functions (Dragicevic et al., 2015). From the VTA dopaminergic neurons project to, among other regions, the hippocampus and the Entorhinal Cortex (Gasbarri et al., 1994). It has been seen that there is an association between DA signaling and modulation of gamma oscillations (Jay, 2003; Weiss et al., 2003; Andersson et al., 2012a; Andersson et al., 2012b; Navakkode et al., 2017). Several studies have shown different effects of DA on hippocampal gamma oscillations, depending on the specific receptor involved in the signaling (for review see Furth et al., 2013).

Although the exact cause of the disease is unknown, several factors seem to contribute to the disruption of the DA signaling and to the formation of the Lewy Bodies, such as inflammation (Joshi and Singh, 2018), oxidative stress (Hwang, 2013), mitochondrial damage (Schapira et al., 1990) and dysfunction of the ubiquitin proteasome system (Dias et al., 2013). The presence of α-synuclein aggregates and the absence of dopaminergic activity lead, in time, to neuronal death (**Figure 5**).

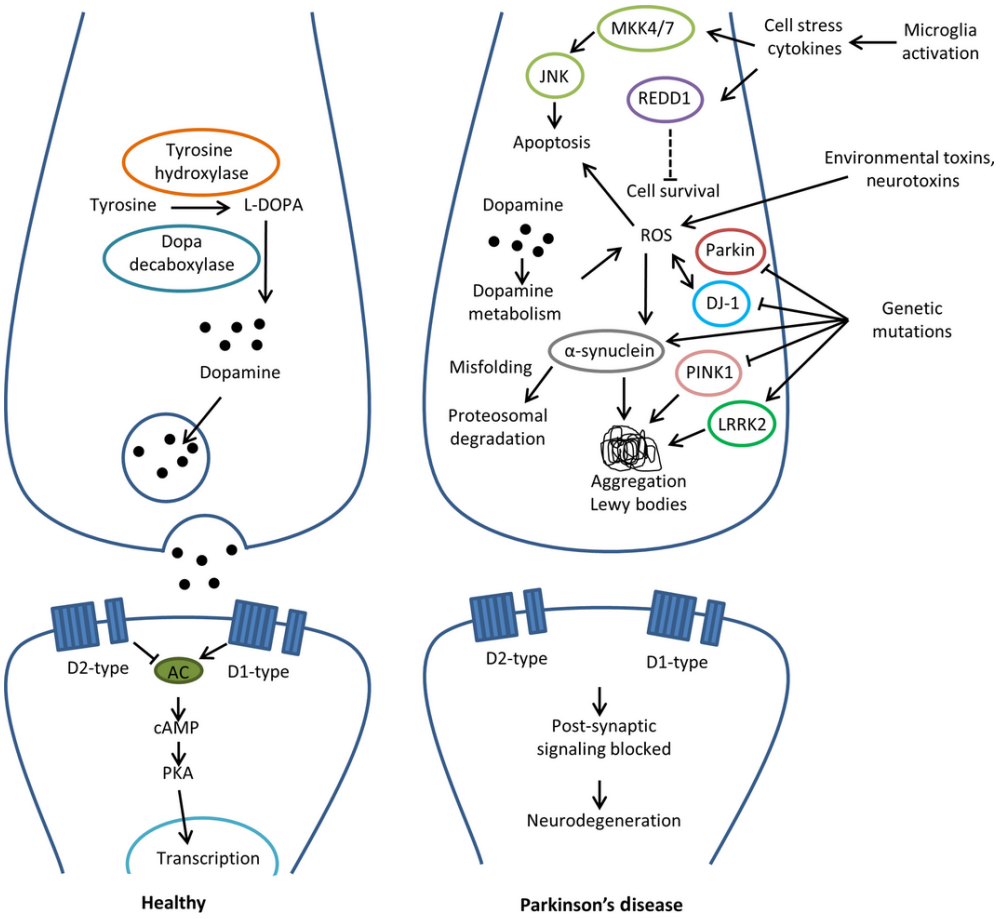


**Figure 5**: The processing of DA in healthy cells (left) and in the case of Parkinson's disease (right). In PD many different factors contribute to the failure of dopamine metabolism, all together leading to the ceasing of post-synaptic signaling and subsequent cell death (Pen and Jensen, 2016).

In the past 40 years PD patients have been treated with Levodopa, a precursor of DA, effective in treating symptoms of the disease by compensating for the decreased DA level, but bringing many adverse effect, especially motor complications, with long-term use. Other treatments involve different DA agonists and, as does Levodopa, come with motor drawbacks. Moreover, as mentioned before, these are only symptomatic treatments and cannot avoid the progression of the disease. Therefore, new strategies are continuously being investigated: fighting the symptoms is not enough and the cause of the disease needs to be unraveled and targeted (Pen and Jensen, 2016).

# 2 AIMS

The overall goal of this thesis is to study cognition-relevant network and cellular mechanisms disturbed in neurodegenerative disorders, in particular Alzheimer's and Parkinson's diseases, and explore potential therapeutic avenues to prevent and/or rescue aberrant network rhythms and their cellular causes as well as behavioral outcomes. Specific aims for each study are:

In **paper I**, investigate whether the deleterious effects of Aβ on cellular, network and cognitive features can be prevented or rescued by activation of the proteasome through the modulation of cellular calcium concentration/dynamics, achieved by selective inhibition of T-type calcium channels.

In **paper II,** test whether pyruvate and/or insulin can correct AD-typical metabolic deficiencies and therefore the downstream network dysfunction in mouse hippocampal slices exposed to Aβ, both at cellular and network level.

In **paper III,** investigate the role of histamine in promoting gamma oscillations in the hippocampus and explore the histamine receptor subtypes and the underlying cellular mechanisms responsible for the transient generation of gamma rhythm.

In **paper IV,** study the effects of the blockage of H3 receptor on circadian activity, recognition memory, anxiety and gamma oscillations in a PD mouse model.

# 3 MATERIALS AND METHODS

This section provides an overview of the methods used in the published articles and manuscripts and an explanation on the rationale and motivation behind their use. A detailed description of the methods can be found in each article and manuscript.

## 3.1 Ethical considerations

All experiments involving animal use were performed in accordance with ethical permits issued to Dr. André Fisahn or to Dr. Gilberto Fisone by the regional ethical board in Sweden (Norra Stockholms Djurförsöksetiska Nämnd; N45/13 and N114/15). Experiments were carefully considered to minimize animal suffering and to reduce the number of animals used.

## 3.2 Choice of experimental models

### 3.2.1 Animal Models

To fill the gap between basic research on neurodegenerative disorders and clinical therapeutics, it is essential to have non-human animal models to increase the knowledge on the pathophysiology and to assess the efficacy of potential treatments. In non-human species, on the other hand, neurodegenerative disorders are an extremely rare condition and recapitulating their complexity in terms of clinical feature is still impossible. These diseases, in fact, are multifactorial and in most cases animal models are not inclusive of all aspects of the disease in question. Animal models have to satisfy several criteria to be considered advantageous models: manageable size, relative costs for maintenance, relatively short time span, relatively easy genetic manipulation and likelihood to generalize studies to other species. Many organisms have been used to mimic parts of different human neurodegenerative disorders, including mouse, rats, zebrafish, *C. elegans* and *Drosophila melanogaster*. Even though each of these species has distinctive advantages for specific experimental questions, for the purpose of this thesis the focus will be on rodents and *Drosophila.*

The mapping of *Drosophila*'s genome in 2000 unraveled striking sequence homology to mammalian genomes (Adams et al., 2000). It has a remarkably short life span and generation time, with a very large number of progeny and it is easy to maintain in terms of space and resources. All these characteristics facilitate the study of many disorders and, in particular, of disorders that manifest late in life, thanks to its short life span. *Drosophila* also offers unique advantages due to its less complicated but well organized CNS: it has neurons and glia, it is protected by a blood-brain barrier and the basic cell biology and major neurotransmitters are

well conserved (Woodruff-Pak, 2008; McGurk et al., 2015). Moreover, pharmacological approaches can be easily used: drugs can be mixed with food, making *Drosophila* a good candidate animal model for screening of therapeutic candidates (Lu and Vogel, 2009).

As a major part of this thesis work is focused on neuronal network function, the model in use was bound to have complex CNS with a neocortex in order to elicit network activity such as gamma oscillations. Rodents represent perfect animal models for this scope. Rodents have become a key model for the study of neurodegenerative disorders because of their relatively short life span and relatively easy genetic manipulation. Rodents are also of great importance in research because they can perform learning and memory tasks, rendering possible to evaluate cognitive, memory and motor function in health and disease.

In **papers I and II** the model used for AD studies is the acute application of amyloid-β to hippocampal slices of C57/BL6N male mice (postnatal 14-30). Acute exposure of $A\beta_{1-42}$ (from now on Aβ) to hippocampal slices (Kurudenkandy et al., 2014) is a well-established method to induce Aβ-related damage to naïve slices and study neuronal network function and cells electrophysiological properties. In **paper III** hippocampal slices from male Sprague-Dawley rats (postnatal 14-22) were used for local field potential and single cell recordings as well as for histology. In **paper IV** a PD mouse model was used: male C57/BL6N were injected with 1 μl of 6-hydroxydopamine (6-OHDA) in each striatum to deplete dopamine neurons (first used as a PD model by Ungerstedt, 1968). Control mice received a sham lesion. These animals were used for both behavioral experiments and electrophysiology.

However, these models can recapitulate just part of the complex scenario of each neurodegenerative disorder and their genetic manipulation cannot mimic the vast majority of cases of neurodegenerative disorders considered in this thesis (AD and PD), which are sporadic.

### 3.2.2 Cell cultures: induced pluripotent stem cells

Since 2006 when Takahasi and Yamanaka proved that mouse skin fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSC; Takahashi and Yamanaka, 2006) the scenario for investigation of pathways involved in different diseases and for testing new possible therapeutic approaches has changed. Despite the use of animal and human embryonic stem cells had and still has a crucial part in drugs screening, it has always been accompanied by ethical concerns. However, given the fact that iPSCs are derived from somatic tissues of patient, the ethical concerns that have slowed down research with human embryonic stem cells have been overcome (Ebert et al., 2012). AD and PD disease models from iPSCs have already been developed and are increasingly used as a model for investigating disease mechanisms (Kondo et al., 2013) and as a drug screening platform (Inoue and Yamanaka, 2011; Yahata et al., 2011). In **paper I** we characterized electrophysiologically iPS cells derived from a patient with familial AD carrying a mutation in the APP gene and reprogrammed into neurons.

## 3.3 Electrophysiology

### 3.3.1 Tissue preparation and maintenance

For electrophysiological recordings, rodents were anesthetized using isoflurane before being sacrificed by decapitation. The brain was dissected and placed in ice-cold artificial cerebrospinal fluid (ACSF). Horizontal sections (350 μm thick) of the ventral hippocampi of both hemispheres were prepared with a vibratome. After cutting, the slices were transferred into a humidified interface holding chamber containing standard ACSF, continuously supplied with humidified carbogen gas, and were allowed to recover for at least 1 hour. Recordings were then carried out in either interface or submerged chambers (see papers). In both cases slices were continuously superfused with ACSF bubbled with carbogen gas, and enriched with different compounds depending on the study (see papers).

### 3.3.2 Local field potential recordings and single-cell experiments

Local field potential (LFP) recordings were performed in interface or submerged chambers according to the nature of the experiments (see papers). Gamma oscillations were induced by superfusing KA (100 nM) or carbachol (20 μM) to the extracellular bath. Gamma oscillations develop in slices in 3-10 minutes after application and are stable after 20 minutes. Recordings at this time point were used as baseline and different compounds were applied after. In other cases, application of various compounds was preceding the induction of oscillations (prevention vs. rescue strategies, see papers). All recordings were carried on *stratum pyramidale* of hippocampal CA3 area, with ACSF-filled borosilicate glass capillaries pulled to a resistance of 3-7 MΩ. Temperature was kept constant throughout the experiment at 34°C in order to maintain stable oscillations.

All single-cell experiments were carried out in a submerged recording chamber. Different intracellular solutions were used according to the configuration of the patch-recordings.

Whole-cell, cell-attached, perforated-patch and single unit configurations were used in different experiments (see papers). Concomitant recordings of pyramidal cells firing and LFP were carried out to relate each AP discharge to a specific phase of the oscillation.

### 3.3.3 iPS cells recordings

Electrophysiological recordings of iPS cells-derived neurons were performed to characterize two different cell lines, one derived from a healthy patient (AF22), the other derived from a patient with familial Alzheimer's disease (ADPII), and to test the action of different compounds. Cells were plated at a density of 50,000 cells per $cm^2$ on glass coverslips to facilitate the passage from the incubator, where they were kept at 37°C with 5% $CO_2$, to the submerged chamber, where whole-cell recordings were performed. Passive neuronal membrane properties (Resting membrane potential (RMP), input resistance ($R_{in}$) and

membrane time constant (τ)) were studied to assay their developmental progression over time. After characterization of the cell lines in control condition between day 36 and day 75 of differentiation, cells were used to test compound treatments (see **Paper I**).

## 3.4 Behavioral experiments with *Drosophila Melanogaster*

Among the many advantages of using *Drosophila* as animal model, there are its standardized behaviors in different scenarios, which can be quantitatively assessed and decline with age and/or pathologies. Flies have the natural tendency to climb as an innate escape response. The climbing assay has proven useful in the study of many neurodegenerative disorders, including Alzheimer's disease (Iijima et al., 2004). Flies exhibit a negative geotaxis response when given a mechanical stimulus. The principle is to place a number of flies in a vial and tap it against a hard surface, strongly enough to cause the flies to fall to the bottom of the vial. As a consequence, flies will orient themselves rapidly and will attempt to climb to the top of the vial, opposed to gravity (Chakraborty et al., 2011; Madabattula et al., 2015). We compared the ability to climb of control and experimental flies (overexpressing human $A\beta_{1-42}$ with Arctic mutation) treated or untreated (see details in **Paper I**) by counting in a set time period how many flies could reach a determined line in the vial. Flies were recorded during the task and videos were analysed to count flies that could cross the set line. This assay, repeated over time, allows also to monitor the progression of the climbing defect, an important feature in the study of neurodegenerative disorders.

## 3.5 Data analysis

All analysis of electrophysiological experiments was made offline using Clampfit 10.7 (Molecular Devices, CA), Mini Analysis (Synaptosoft Inc), Axograph X (Berkley, CA), Kaleidagraph (Synergy Software), MATLAB (Mathworks, Matick, MA) or IGOR Pro (WaveMetrics). Statistical analysis was carried out in GraphPad Prism (GraphPad Software, Inc).

Fast Fourier Transform for power spectra was calculated from 60 seconds long LFP recordings using Axograph. Gamma power was calculated as the integrated power spectrum between 30 and 80 Hz using Kaleidagraph. In some figures, the data shown are normalized to the baseline of KA-induced oscillations, but statistical tests were performed using the raw data. In **paper I** spectrograms were made for visualization purpose using MATLAB but were not used for any analysis.

Synaptic currents were detected offline using Mini Analysis software or custom-made macros in IGOR Pro. Charge transfer, amplitude and inter-event-interval (IEI) were analysed using GraphPad Prism with the results representing average values taken over 60 seconds periods.

Spike phase-coupling analysis was performed on concomitant LFP recordings and single-cell recordings using a custom-made routine in MATLAB to relate the pyramidal cells spiking activity to ongoing gamma oscillations (Andersson et al., 2012). LFP traces were previously filtered in the gamma-band frequencies and APs were detected using an amplitude threshold. Using a Hilbert transform, the angle of the oscillation at which each AP occurs can be determined. Each AP is represented by a vector of length 1 with an angle corresponding to the phase of the oscillation at which the AP occurred. The vectors were then averaged and the resultant vector describes the preferred phase of firing (phase-angle) and how recurrent the firing in that phase is (vector length). The peak of the oscillation cycle corresponds to 0 and the trough to $\pm\pi$ in the polar plots, with all the phase-angles distributed accordingly.

Data analysis for iPS cells recordings was performed using Clampfit 10.7. RMP, $R_{in}$, $\tau$ and percentage of cells in which AP occurred were examined. The $R_{in}$ was calculated from voltage-clamp steps recordings and derived from the linear portion of the current-voltage plot. All the parameters were analysed using GraphPad Prism.

All data are represented as mean ± standard error of mean (SEM).

# 4 RESULTS AND DISCUSSION

## 4.1 Paper I

Altered calcium homeostasis is one of the consequences of Aβ accumulation in AD. In **paper I** we show that the inhibition of T-type calcium channels has beneficial effects in several models of AD and can restore cellular and network function impaired by Aβ, probably through the activation of the ubiquitin-proteasome system.

Disturbance in the protein degradation mechanisms is common in many human diseases such as cancer (Devoy et al., 2005; Jara et al., 2013), immunological disorders (Wang and Maldonado, 2006) and neurodegenerative diseases (Dantuma and Bott, 2014; Zheng et al., 2016). The impairment in the ubiquitin-proteasome system (UPS) in AD is well established (Keller et al., 2000; Hong 2014) and can be a target for potential therapeutic strategies (Qing et al., 2004; Gadhave et al., 2016).

This study began in collaboration with a biotech company through which we had access to 14 FDA-approved drugs all sharing the ability to increase proteolysis (Leestemaker et al., 2017). One of these drugs, Pimozide, is the object of study in this paper.

To evaluate Pimozide, we initially tested its effects on a *Drosophila Melanogaster* model of AD, created by Crowther and colleagues as a drug-testing tool (Crowther et al., 2005). The aim of these experiments was to understand if this drug could have any functional benefits and/or could decrease the burden of Aβ aggregates in a very severe model of the disease in a short time. The behavioral test (climbing test) showed a clear improvement of the animals treated with Pimozide over sham treated flies. The genetic controls used and treated with the drug showed no adverse effects. The reason behind the improvement in the motor ability of the treated flies was revealed by immunohistological studies on whole brains stained for Aβ. We observed a significant reduction of the size and number of Aβ aggregates in treated flies compared to the sham treated ones.

With these first very encouraging results, we set out to study Pimozide's effect on a model that could give us more insight into the cognitive decline typical of AD. As said previously, cognitive decline is strictly related to gamma oscillations disruption in many neurodegenerative disorders. Gamma oscillations can be studied both *in vivo* and *in vitro*: in *in vitro* studies they represent a useful tool to test effects of compounds on a cognition-relevant network activity, and they could be a remarkable functional biomarker for diagnosis if studied on patients.

The effect of Aβ on KA-induced gamma oscillations has been widely explored (Kurudenkandy et al., 2014) and some results were replicated in this study as controls to the compound's treatment. We performed experiments to evaluate potential preventive effects of Pimozide for Aβ-induced degradation of gamma oscillations, and we found that the drug was

effective in preventing the degradation in a concentration-dependent manner. With these promising preventive results we started wondering what the mechanism behind Pimozide's action could be. Pimozide is known to act on several receptor subtypes as an inhibitor or an antagonist. To dissect the mechanism responsible for its actions, we tested several specific inhibitors for receptors or channels known to be targeted by Pimozide. The only other compound able to reproduce the same effect observed with Pimozide on Aβ-disrupted gamma oscillations was the specific T-type calcium channel inhibitor, Penfluridol. This result is in accordance with the alterations in the calcium homeostasis in AD that have been studied since the '90s (Mattson et al., 1992, 1993; Arispe et al., 1993) and are known to be a central issue in the disease. However the specificity to T-type calcium channels is novel.

Although the preventive effects of these T-type calcium channel inhibitors are of importance, the definition of "preventive" itself renders them less valuable from a clinical perspective. In a disease such as AD, in which pathologic changes occur several years before the clinical symptoms and there is no effective early-diagnosis tool, a preventive drug would be, unfortunately, of little use. We therefore tested the two T-type calcium channel inhibitors (Pimozide and Penfluridol) on hippocampal slices previously treated with Aβ to evaluate their potential restorative effect on gamma oscillations; we observed a partial rescue over time with both compounds and with two additional T-type calcium channel inhibitors with a different chemical structure, NNC55-0396 and ML-218.

Knowing that several cellular and synaptic mechanisms can underlie the changes in gamma oscillation power, we proceeded to examine the balance between excitatory and inhibitory synaptic activity and the phase-synchronized AP firing (Fisahn et al., 2004; Leão et al., 2009). First, we studied the synaptic properties of CA3 pyramidal cells. It has been previously shown that Aβ changes the equilibrium between excitation and inhibition (Kurudenkandy et al., 2014). To test whether T-type calcium channel inhibition could restore this balance, we recorded excitatory (EPSCs) and inhibitory (IPSCs) postsynaptic currents in slices activated with KA. Both Pimozide and Penfluridol proved to be effective in restoring the Aβ-induced increase of EPSCs' amplitude and frequency to their normal levels, while they were ineffective on reduced IPSCs, where the decrease initiated by Aβ continued over time even in presence of either of the two inhibitors. This selective effect on synaptic properties may be the explanation for the partial rescue of disrupted gamma oscillations seen in our previous experiments.

Secondly, we performed single unit recordings of pyramidal cells concomitant with LFP recordings in hippocampal slices activated with KA. The acute application of Aβ resulted in, as seen before, a severe reduction of gamma oscillations in concomitance with an increase in APs firing frequency of PC. Spike-phase coupling was also evaluated before and after Aβ treatment. We found a substantial desynchronization of AP firing in relation to the gamma cycle and a shift in the AP preferred phase-angle. After the application of Aβ we applied in 2 separate sets of experiments Pimozide and Penfluridol. In both cases we found that, in addition to the partial gamma oscillations rescue seen before, the AP firing frequency

decreased back to normal level. Moreover, analysing the AP spike-phase coupling after compound applications we observed that the treatments had completely rescued the desynchronization of AP firing and the shift in the AP preferred phase-angle.

To bring this study a step further toward the complexity of the human disease, we took advantage of the relatively new iPS cell technology. These cells can recapitulate diseases in a dish and can be extremely useful to test drugs. For this reason we electrophysiologically characterized one line of iPS cells derived from a healthy patient (AF22) and one line derived from a patient with familial AD (APP mutation, ADPII). We monitored the passive membrane properties and firing ability of these two lines for 40 days, between day 35 and day 75 of differentiation. The comparison of the different parameters of these two lines revealed that although during the differentiation process the AD cell line matures faster than the control, it also degenerates faster. However, a 48-hour treatment of the ADPII line with Penfluridol or Pimozide toward the end of our time frame, when the degeneration was already ongoing, turned out to be effective in bringing all the passive membrane properties and firing frequency to levels similar to those of the AF22 line. Further basic characterization of these cell lines needs to be done and is ongoing. Moreover, further investigation of the effects of the two T-type calcium channel inhibitors on this model is necessary since our initial results are very encouraging.

Although calcium has been proven of importance in the activation of the proteasome machinery (Djackovic et al., 2009), the excess of calcium influx in the cells due to Aβ is deleterious and creates a vicious cycle (LaFerla, 2002). However, there are some controversies in the field. Some studies suggest the blockage of calcium channels as a therapeutic strategy for AD (for review, Yu et al., 2009), and most of them are focused on the L-type calcium channels (Yagami et al., 2004; Anekonda et al., 2011; Paris et al., 2011). The selectivity of the available T-type calcium channel blockers and, even more, L-type calcium channel blockers doesn't allow for a definitive distinction between the two. Most of them have overlapping effects, with slight preferences toward one of the two channel types. Although Pimozide and Penfluridol are not very specific drugs, the use in this paper of the more selective and structurally different NNC55-0396 and ML-218 proves the link between the gain-of-function we see and T-type calcium channels.

Overall, this study highlights the importance of the dysregulation of calcium homeostasis in relation to the proteasome machinery in AD, and offers a potential avenue toward its restoration through the inhibition of T-type calcium channels.

## 4.2 Paper II

In this paper we discuss the connection between Aβ and hypometabolism and a potential combinational treatment acting on reduced glucose metabolism and insulin resistance.

Among the earliest symptoms of AD, hypometabolism has a central role. It manifests as changed glucose consumption (Jin et al., 2013), neuronal energy deficit and oxidative stress (Gella and Durany, 2009). Glucose metabolism is reduced by ~50% in sporadic AD, while it seems to be not so much affected in familial AD (Hoyer, 1992). Hypometabolism itself seems to contribute to production and accumulation of Aβ (Velliquette et al., 2005; Koike et al., 2010), which in turn leads to many downstream effects including insulin resistance (Xie et al., 2002; Biessels and Reagan, 2015), further glucose consumption impairment (Carrano et al., 2011), hyperactivity of neurons, astrocytes and microglia as well as calcium homeostasis dysregulation (Brawek and Garaschuk, 2014).

We have previously shown that AD-related network hyperexcitability is due to a depolarizing effect of Aβ, both in *ex-vivo* hippocampal slices from transgenic mice (Zilberter et al., 2013) and from wild-type hippocampal pyramidal cells during acute Aβ application (Kurudenkandy et al., 2014). However, in our recordings from CA3 pyramidal cells in whole-cell configuration we did not see any membrane potential depolarization, seen as a lack of changes in the holding current at -70 mV. This was surprising and intriguing and made us wonder if the reason of this difference could be a technical issue. In fact, the previous results were obtained either using completely non-invasive cell-attached recordings or the relatively non-invasive perforated-patch configuration. In both cases, the intracellular make-up remains unaltered. The intracellular solution used for whole-cell recordings contained 2 mM $Na_2$-ATP: in this configuration, the recording pipette solution can diffuse inside the recorded cell, compensating for a potential lack of energy and preventing the Aβ-induced depolarization. Successive experiments with different concentration of ATP in the intracellular solution proved that when the external energy source was lowered, the deleterious effect of Aβ presented itself again. Moreover, if the ATP concentration was considerably reduced the depolarization of the recorded cell occurred even in absence of Aβ. Therefore, we concluded that Aβ depolarizes pyramidal cells by inducing an intracellular ATP shortage. This deficit can have many effects on all ATP-dependent processes in the neurons: for instance, the depolarization of the membrane could be due to the malfunctioning of the $Na^+/K^+$ ATPase, responsible for the maintenance of the resting membrane potential.

Another effect of Aβ on neurons is insulin resistance (Xie et al., 2002; Hoyer, 2004; Pearson-Leary and McNay, 2012). Physiologically, insulin increases glucose uptake into cells (Leney and Tavaré, 2009) by inducing the translocation of glucose transporters (GluTs) to the plasma membrane. Insulin resistance could therefore bring to a decrease of this translocation with consequent glucose metabolism impairment (Pearson-Leary and McNay, 2012; Jin et al., 2013). With western-blot analysis we checked the levels of the insulin-dependent glucose transporter GluT4 and insulin-independent GluT1 and GluT3 in activated hippocampal slices (carbachol, 20 μM) from WT mice in control condition and after acute Aβ application. We found no changes in membrane expression of any glucose transporters after treatment with Aβ. In accordance with our results, other studies have revealed no changes in the expression of GluTs in different AD mouse models (Hooijmans et al., 2007; Chen et al., 2014). Also, post-mortem brain studies have revealed no changes in the expression of GluT4 in AD

patients (Steen et al., 2005) and no changes in glucose uptake by insulin administration (Talbot et al., 2012). Together, these results suggest that glucose transport is not involved in Aβ toxicity on neuronal energy metabolism.

The decreased glucose metabolism, with consequent ATP shortage, and the insulin resistance induced by accumulation of Aβ have very important role in the pathogenesis of AD. We hypothesized that acting on these two processes could stop the vicious cycle of AD progression. To counteract energy deficiency we used pyruvate, the principal mitochondrial source of energy and potent ROS scavenger; to counteract insulin resistance we used insulin. In perforated-patch experiments we observed that the addition of pyruvate to the bath solution completely prevented the depolarization of CA3 hippocampal pyramidal cells induced by Aβ. Insulin perfusion before Aβ application also prevented the depolarization, though not completely.

As said for **Paper I**, in presence of Aβ there is a shift in the neuronal network excitation/inhibition balance (Kurudenkandy et al., 2014) seen as a decrease in IPSCs amplitude and frequency and an increase in EPSCs frequency in activated slices. Presence of pyruvate in the bath completely prevented the Aβ-induced frequency increase of EPSCs, while it had no effects on the Aβ-induced reduction of IPSCs' frequency or amplitude. Conversely, insulin presence before Aβ application was effective in preventing completely the Aβ effects on IPSCs but had only a weak effect on EPSCs. Since it has been reported that insulin-like growth factor 1 (IGF-1) receptor signaling regulates differently spontaneous and evoked excitatory transmission in hippocampal synapses (Gazit et al., 2016), we studied miniature EPSCs (mEPSCs) as well. This revealed a different scenario: insulin had a very strong effect on mEPSCs (in line with what reported by Gazit and colleagues (2016)), maintaining them at control level in presence of Aβ, while pyruvate had a partial preventive effect.

Moreover, we studied gamma oscillations: we found that the detrimental effect caused by acute Aβ application on hippocampal slices is also evident in carbachol-induced gamma oscillations. In control conditions, neither insulin nor pyruvate showed any effects on gamma oscillations. However, when perfused after Aβ application, they both partially restored the disrupted gamma power. The restorative effect of insulin on gamma oscillations was blocked by the presence of insulin receptor antagonist, suggesting that insulin's effect is mediated by insulin receptors rather than IGF-1 receptors.

All these results seem to support the idea that insulin and pyruvate normalize the Aβ effects through different pathways. Since these effects are in part non-overlapping, the use of the two compounds synergistically could be an effective treatment for the several deleterious effects exerted by Aβ. In fact, the application of pyruvate and insulin combined (PIN) on Aβ-disrupted gamma oscillations completely recovered their power.

We suggest that acting on the pathways leading to Aβ accumulation might be a good strategy in the fight against AD. However, because AD is a disease with many different intricate risk

factors and cascade events, the counteraction of any of them alone might not be sufficient. In this paper we show that a synergistic compensation of two main damaged pathways is effective in restoring cognition-relevant network rhythmicity and the responsible cellular parameters.

## 4.3 Paper III

In this paper we show that the activation of histamine receptors generates transient gamma oscillations in the area CA3 of the hippocampus, in a dose-dependent manner, and that this effect is dependent on H1 receptor.

Apart from the role of histamine in mediating allergic reactions, gastric acid secretion and inflammation, histamine also has an important role as a neurotransmitter in the CNS. The central histamine system is involved in many brain functions such as wakefulness, arousal, control of pituitary hormone secretion, attention and cognitive functions. The histaminergic neurons originate from the tuberomamillary nucleus in the dorsal hypothalamus and they send projections to many areas of the brain (Garbarg et al., 1974), including pyramidal cells in area CA3 and CA2 of the hippocampus (Maglóczky et al., 1994).

The action of histamine in the brain is mediated by four histamine receptors (H1-H4), all of which are G-coupled receptors. H1 and H2 are largely excitatory, being coupled to $G_q$ and $G_s$ proteins respectively (Black et al., 1972); H3 and H4, on the other hand, are mostly inhibitory, being coupled to $G_{i/o}$ proteins (Arrang et al., 1983; Nguyen et al., 2001). However, H4 receptor is so poorly present in the CNS that its very existence has been debated for a long time (Strakhova et al., 2009); its physiological effects, moreover, are still poorly understood.

We started this study by examining the distribution of histamine receptor subtypes in the hippocampus of young (18 days postnatal) and adult (6 months old) rats. Since there are no available antibodies specific for each histamine receptor subtype, we used *in situ* hybridization probes targeting mRNA of H1, H2, H3 and H4 receptors to study their gene expression. We observed that H1, H2 and H3 receptor are highly expressed in various areas of the hippocampus, with small differences related to the age of the animal, while H4 expression could not be detected. Moreover, H1, H2 and H3 were mostly expressed in *stratum pyramidale* of CA3.

We then confirmed that the application of histamine depolarizes CA3 pyramidal cells, as previously reported (Yanovsky and Haas, 1998). In our hands, histamine induced a depolarization of the resting membrane potential of pyramidal cells, an increase in their membrane resistance and a decrease of the rheobase current (amount of current needed to trigger an action potential). It also induced a conspicuous increment of the spontaneous firing of PC. Similar results were found on FS-IN, which doubled their action potential firing rate in presence of histamine. Because histamine was able to excite both PC and FS-IN we

hypothesized that it might have an effect on the network activity as well. In fact, the application of histamine on quiescent hippocampal slices resulted in the generation of gamma oscillations whose power quickly increased within the first 60 seconds after application, and then gradually decreased in a 10 minutes time frame. These transient gamma oscillations generated by histamine are dose dependent, and the use of excessively high concentration of histamine resulted in PC depolarization block and failure to generate gamma oscillations.

After having established that histaminergic rhythmogenesis was due to the direct action of histamine on CA3 neurons (see paper), we set out to establish through which receptor subtype histamine was inducing the generation of gamma oscillations. By blocking each histamine receptor with its specific antagonist, we established that H1 receptors are necessary to induce network activity in the gamma frequency range, and H3 receptor might be involved as regulators of histamine-induced activity. H1 receptors have been reported to inhibit "leak" potassium currents (Haas et al., 2008), promoting neurons excitability. Given that the H1 receptor is coupled to $G_{q/11}$, which is known to inhibit one type of potassium channel, KCNQ channels (Brown and Passmore, 2009), we decided to investigate the possible role of these channels in the histaminergic rhythmogenesis. KCNQ channels are important for the maintenance of the resting membrane potential and are expressed in both PC and FS-IN; they are slow to activate and deactivate and they do not inactivate. We could therefore study the tail currents after depolarization steps. We observed that the application of histamine reduces these tail currents in PC, while in FS-IN its application does not induce any changes. However, in FS-IN we observed little or no tail current to begin with. The use of a selective inhibitor of the KCNQ channels did not generate on its own gamma oscillations, but the application of a KCNQ channel opener counteracted the generation of gamma oscillations induced by histamine. Therefore, it seems that KCNQ channels inhibition is necessary but not sufficient for histamine-induced gamma oscillations.

As said previously, the generation of gamma oscillations depends both on the excitability of pyramidal cells and FS-IN and on efficient synaptic signaling. We therefore studied EPSCs and IPSCs in pyramidal cells. We did not see any changes in the excitatory synaptic currents' parameters in presence of histamine. As for IPSCs, no changes in the total charge transfer were detected but the analysis of their spectral power in the gamma-band revealed a significant increase in presence of histamine.

We next examined the phase-preference of action potential firing of PC and FS-IN during histamine-induced gamma oscillations. By comparing the phase angle of PC to that of FS-IN we could establish that pyramidal cells fire significantly earlier than fast spiking interneurons in each cycle, consistently with what has been previously described (Fisahn et al., 1998; Tukker et al., 2007; Buzsáki and Wang, 2012).

In summary, in this study we describe a new physiological mechanism to generate gamma oscillations in the hippocampus that seems to be dependent on the inhibition of KCNQ channels.

## 4.4 Paper IV

While in **paper III** the focus was on H1 receptor, in this study H3 receptors are investigated as potential target for the treatment of circadian rhythm and cognitive dysfunction in a mouse model of Parkinson's disease.

As mentioned in the introduction, the non-motor symptoms of PD are getting increasingly more attention for being a main issue in the disease. At the same time, the role of histamine in the basal ganglia physiology and in the pathophysiology of PD is an emerging question. Post-mortem studies have revealed altered levels of histamine in different neurological and psychiatric disorders, and specifically abnormally high concentrations in PD patients (Rinne et al., 2002); histaminergic neurons are also known to regulate wakefulness through their pacemaker-like firing pattern in the tuberomamillary nucleus (Taddese and Bean 2002). Of importance, especially for sleep related symptoms of Parkinson's disease, is the H3 receptor. It was originally described in 1983 as a presynaptic autoreceptor, responsible for the inhibition of histamine release in the brain (Arrang et al., 1983) and, later on, it was shown to have a role as a heteroreceptor as well (Blandina et al., 1996; Schlicker et al., 1998). Antagonizing pharmacologically the H3 receptor has been proven effective in counteracting narcolepsy (Lin et al., 2008; Guo et al., 2009; Lin et al., 2011).

In this paper we compare mice in which depletion of DA neurons was induced by injecting 6-OHDA in each striatum and control mice that received a sham bilateral lesion. To study the effects of the lesion, mice were first monitored during 12h:12h light/dark cycle, and we observed that 6-OHDA-lesion mice exhibited reduced spontaneous motor activity during the dark phase (active phase) of the 24-hour cycle compared to control mice. Moreover, the following maintenance of these mice in constant darkness for 21 days showed a disruption in the endogenous circadian rhythm: 6-OHDA-lesion mice showed undefined rest/activity periods and overall lowered activity levels, in contrast with sham mice who maintained their normal activity pattern during day and night. We then tested the effects of thioperamide, a selective H3 antagonist (Arrang et al., 1987), on the disrupted circadian rhythm in 6-OHDA and sham mice in a 12h:12h light/dark cycle (see timeline on paper). The administration of thioperamide restored the normal rest/activity distribution in 6-OHDA-lesion mice.

To evaluate the effects of thioperamide on cognitive impairment induced by 6-OHDA-lesion, we studied 1) the novel object recognition test performance of treated mice; 2) *in vitro* gamma oscillations power changes on hippocampal slices from injected and treated animals. Animals with 6-OHDA-lesion were confirmed to be unable to recognize novel objects after a familiarization phase, as proven previously (Bonito-Oliva et al., 2014). This deficit was, however, reversed with the administration of thioperamide.

Gamma oscillations have been shown to be degraded in non-demented PD patients, underlying their cognitive decline (Olde Dubbelink et al., 2013). Sham- and 6-OHDA-lesion mice were sacrificed and hippocampal slices were obtained (see paper). Gamma oscillations were induced with KA and LFP were recorded in area CA3 of the hippocampus. Stable

oscillations recorded from sham mice injected with saline were indistinguishable from gamma oscillations recorded from naïve mice, while gamma oscillations recorded from 6-OHDA-lesion mice were severely disrupted. In slices obtained from 6-OHDA-lesion mice injected with thioperamide, however, the power of gamma oscillations recorded was restored to control levels. The reduction of gamma oscillations in 6-OHDA-lesion mice indicates that dopaminergic innervation of the hippocampus is compromised, and could explain the impairment of long-term novel object recognition. It has been reported that the activation of H3 reduced the power of gamma oscillations induced by KA on WT hippocampal slices (Andersson et al., 2010). However, how thioperamide rescues the power is still to be elucidated. The H3 receptor mediates presynaptic inhibition of several neurotransmitters release, including dopamine (Schlicker et al., 1994; Hill et al., 1997; Brown et al., 2001). Its inhibition, therefore, might remove the inhibition on D1, thus facilitating the dopamine transmission. The inhibition of H3 receptors with the antagonist GSK189254 was reported to increase acetylcholine, dopamine and noradrenaline levels in the anterior cingulate cortex and acetylcholine in the hippocampus, and was suggested to improve cognitive performance in AD (Medhurst et al., 2007). Interestingly, in a recently published study, non-motor symptoms (circadian rhythm and cognitive performance) in a mouse model of Huntington's disease (where dopaminergic pathways are also compromised) were ameliorated with the antagonist GSK189254 (Whittaker et al., 2017).

In summary, this study highlights the importance of H3 receptor antagonists as potential treatments for cognitive impairment and circadian rhythm dysregulation associated with PD.

# 5 CONCLUSIONS

The intriguing ability of the brain to generate electrical oscillations through the synchronized activity of neurons is extremely relevant in both physiology and pathology of the brain. Brain rhythms are very well conserved across different species (Buzsáki et al., 2013). In particular, frequency bands and temporal aspects of human oscillations (with their respective behavioral correlates) can be found in many other mammals (Buzsáki et al., 2013), making them important for translational research. The study of non-invasive EEG recordings can be linked to pre-clinical research. This is of particular relevance in pathologies, where the alteration of brain rhythms in patients can be studied in detail in research animals *in vitro* and can potentially lead to the identification of the pathophysiological mechanisms responsible for the alterations. *In vitro* preparations are ideal for pharmacological manipulation and give direct visual controls of the area or cells of interest for the recordings (Teyler, 1980). However, even though many conditions can be controlled during electrical recordings in brain slices, the slicing process itself damages the tissue, removing it from the blood supply and truncating many inputs coming from distant brain regions. Moreover, even though the disease models used in this thesis are well validated and broadly used, they do not reproduce all aspects of the diseases in question. Further *in vivo* experiments are necessary to continue these studies starting from behavioral experiments in animal models (already initiated in the case of **papers II** and **IV**, in preparation for **paper I**). Moreover, for all the studies described here more detailed experiments on the role of other cell types important for cognition (various interneuron sub-types, astrocytes, microglia) will need to be performed.

The various compounds used in these projects to prevent or rescue neuronal damage have possible clinical applications. For AD, the two different approaches use FDA-approved drugs (in the case of T-type calcium channel inhibitors) or insulin and pyruvate, compounds known to be not harmful if potentially used in patients. Moreover, they are all known to cross the blood-brain barrier. The use of these compounds in *in vitro* models gave promising results in counteracting the effects of Aβ at different system levels. The two approaches act on mechanisms that are common to other neurodegenerative diseases as well, including PD (Yang et al., 2014; Anandhan et al., 2017; Athauda and Foltynie, 2016) and potentially Huntington's disease (HD) (Giacomello et al., 2013; Lalić et al., 2008). Similar studies to those presented in this thesis are already planned in our lab in PD and HD mouse models as well as on iPS cells from PD and HD patients.

On the other hand, the presented study on PD investigates the role of histamine in cognition-relevant network rhythms and at a behavioral level in mice. The neuronal histaminergic system is involved in many physiological functions (potentially also in the *in vivo* generation of gamma oscillations, as examined in **paper III**) and is known to be disrupted in many neurodegenerative disorders. The modulation of histamine levels or modulation of histamine receptors might be useful in the fight against AD and PD.

In conclusion, the studies included in this thesis provide new insight into mechanisms potentially involved in *in vivo* gamma oscillations generation and mechanisms behind the disruption of gamma oscillations (and their linked cognitive deficiencies) in neurodegenerative diseases and suggest potential treatment strategies to prevent and rescue these deficiencies.

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